REMARKS

Claims 1 to 30 are pending.

The specification has been amended to correct various typographical errors and to add a Cross-Reference to Related Applications section. Accordingly, no new matter has been introduced by way of these amendments.

Applicants note that some of the page references used by the Examiner appear to correspond to a related previous application, USSN 09/339,632. The instant application is a continuation of PCT/US99/27505, which is a continuation-in-part of USSN 09/339,632, and as a result the pagination in the instant application is different from that of USSN 09/339,632. Applicants have tried to address the appropriate rejections based on assumptions that in some instances the page numbers being referred to are from USSN 09/339,632. However, if any such assumptions are incorrect, Applicants respectfully request clarification. All page numbers from the specification that are referred to in this response are from the instant application.

This invention is directed to an *in vitro* cultured tissue construct of cultured cells and endogenously produced extracellular matrix components. The invention is also directed to the method for producing this cultured tissue construct.

Prior art tissue constructs were constructed with a variety of techniques, but all of these prior art techniques employed either exogenous matrix components or synthetic members during the culturing conditions, or both. (See specification, page 3, lines 5-7, "Heretofore, current engineered living tissue constructs are not completely cell assembled and must rely on either the addition or incorporation of exogenous matrix components or synthetic members for structure or support, or both.")

For instance, one of the cited prior art references, Bell, U.S. 4,485,096, teaches how to make a skin-equivalent by growing keratinocytes on contracted collagen lattices. Bell teaches that keratinocytes can be plated at the time the matrix gel forms, at any time while the lattice is contracting, or at any time after the contraction is completed (col. 4, lines 19-22). The keratinocytes form a confluent layer on the lattice surface (col. 4, line 24). This teaching shows the prior art reliance on exogenous matrix components.

The claims to the cultured tissue construct are written to describe this feature of the invention.

I. <u>Rejections Under Section 112, First Paragraph.</u>

Claims 1-30 are rejected under Section 112, first paragraph, for a variety of reasons. Each of these rejections will be addressed in turn.

A. Lack of an enabling disclosure for lack of PEG or plates with collagen treated PTFE membrane.

The Examiner states that the specification fails to provide an enabling disclosure for a tissue construct and the method of its production in the absence of synthetic members during culturing conditions. Applicants respectfully traverse this rejection.

While the Examiner refers to the specification and the examples using polyethylene glycol ("PEG") in the culture media, this is not a "synthetic member." The specification indicates at page 16, line 20 to page 17, line 5 that PEG may be provided in solution to the media, provided that the concentration is at about 5% w/v or less, to assist in matrix processing and deposition. The supplementation with a neutral polymer is optional. The specification also indicates that the cell-matrix constructs of the invention may be produced without such a neutral polymer. (page 16, lines 21-22). In addition, constructs discussed in Example 15, using conditions 2 and 3, and Example 16 were prepared without PEG.

The Examiner also refers to the use of a Transwell six-well tray which is coated with a collagen-treated PTFE membrane. The Transwell plates are available with and without a collagen coating. The Examiner has pointed to the collagen coated product line, not the polycarbonate or polyester lines that are not coated. The specification indicates at page 10, lines 7-9 that for human dermal fibroblasts, the most preferred material is polycarbonate.

Thus, it would not require undue experimentation to make and use a tissue construct in the method disclosed without the use of PEG or without a tray with a collagen-treated PTFE membrane. For these reasons, the Examiner is requested to reconsider the rejections and to withdraw them.

B. Lack of an enabling disclosure for morphology.

The Examiner states that the specification is not enabling for a tissue construct and the method of producing such, which exhibits an extracellular matrix comprising fibrillar collagen showing a packing organization of fibrils and fibril bundles exhibiting a quarter-staggered 67 nm banding pattern and the presence of decorin.

Applicants respectfully traverse this rejection. Applicants are not required to provide this type of proof when one of skill in the art would believe and accept it. In addition, the specification states at page 4, lines 14-17, that standard immunohistochemistry techniques as well as TEM can be used to determine the presence of or visualize decorin.

In addition, Applicants enclose herewith a Rule 132 Declaration of Nancy Parenteau, which provides evidence indicating that the extracellular matrix of a tissue construct produced in the absence of exogenous matrix components contains collagen fibrils which exhibit a quarter-staggered 67 nm banding pattern and decorin.

Therefore, the Examiner is requested to reconsider and to withdraw this rejection.

✓ C. Lack of enabling disclosure for chemically defined media and with no non-human components.

The Examiner states that the specification fails to provide an enabling disclosure for a cultured tissue construct or the method of producing such utilizing cells cultured in chemically defined media and with no non-human components. Applicants respectfully traverse this rejection.

Although some of the examples do use newborn calf serum in the growth medium, as in Example 1 on page 26, lines 6-7, Example 3 was conducted without newborn calf serum (page 30, line 23). The histological evaluation in Example 3 demonstrated that the constructs grown in defined medium were similar to those grown in the presence of 2% newborn calf serum (page 31, lines 7-8). This statement compares the serum free constructs of Example 3 to those made with serum in the culture medium. Besides endogenously produced fibrillar collagen, decorin and glycosaminoglycan were also present in the cell-matrix construct. (page 31, lines 13-14). Examples 3, 4, 15, and 16 are serum-free examples. Examples 3 and 15 are directed to dermal (or connective tissue) constructs, while Examples 4 and 16 are directed to bilayer skin constructs.

These concepts are discussed at page 11, line 25 to page 12, line 12.

> The use of chemically defined culture media is preferred, that is, media free of undefined animal organ or tissue extracts, for example, serum, pituitary extract, hypothalamic extract, placental extract, or embryonic extract or proteins and factors secreted by feeder cells. In a most preferred embodiment, the media is free of undefined components and defined biological components derived from non-human sources. Although the addition of undefined components is not preferred, they may be used in accordance with the disclosed methods at any point in culture in order to fabricate successfully a tissue construct. When the invention is carried out utilizing screened human cells cultured using chemically defined components derived from no non-human animal sources, the resultant tissue construct is a defined human tissue construct. Synthetic functional equivalents may also be added to supplement chemically defined media within the purview of the definition of chemically defined for use in the most preferred fabrication method. Generally, one of skill in the art of cell culture will be able to determine suitable natural human, human recombinant, or synthetic equivalents to commonly known animal components to supplement the culture media of the invention without undue investigation or experimentation. The advantages in using such a construct in the clinic is that the concern of adventitious animal or cross-species virus contamination and infection is diminished. In the testing scenario, the advantages of a chemically defined construct is that when tested, there is no chance of the results being confounded due to the presence of the undefined components.

For these reasons, the Examiner is requested to reconsider and to withdraw this rejection.

D. Claims 4, 5, 13, and 14. Dermal papilla of hair follicles.

The Examiner states that the specification fails to provide an enabling disclosure for a cultured tissue construct comprising cultured cells from the dermal papilla of hair follicles which are grown to produce a layer of extracellular matrix, specifically that the presence of collagen, decorin or glycosaminoglycan is not disclosed. Applicants respectfully traverse this rejection.

The dermal papilla cells are locally seeded on the cell-matrix and were in turn seeded with keratinocytes as shown in Example 12. The specification states at page 42, lines 2-6, that the "resulting skin constructs exhibited the basic morphological organization similar to skin: a dermal layer consisting of fibroblasts surrounded by endogenously produced matrix, including endogenously produced fibrillar collagen, decorin, and glycosaminoglycan, localized areas of dermal papilla cells and a continuous, stratified layer of keratinocytes across the cell-matrix construct and the dermal papillae." Thus, the specification does disclose that collagen, decorin, and GAG existed within the tissue.

For these reasons, the Examiner is requested to reconsider and to withdraw the rejection.

E. Claim 15. Three cell layer tissue construct.

The Examiner states that the specification does not provide an exemplifying disclosure for a method of making a three cell layer tissue construct. (The Examiner notes that the above referenced Example 12 does not technically comprise three layers of cells, but two layers with seeding of a third cell type within the first layer).

Applicants respectfully traverse this rejection. Once one of skill in the art has created a tissue construct of two layers, it would be possible to seed cells onto the two-layer construct, which cells would then grow into an additional third layer on the construct, thus creating a three layer tissue construct without undue experimentation.

For these reasons, the Examiner is requested to reconsider the rejection and to withdraw it.

F. Genetically modified cells.

The Examiner states that the specification fails to provide an enabling disclosure for any genetically modified fibroblast cells other than the exemplified human dermal fibroblasts transfected to express platelet derived growth factor.

Applicants respectfully traverse this rejection. The genetic engineering of cells is within the skill in the art. Those of skill in the art have been genetically engineering human cells for a number of years. This is even shown by the Examiner's citation of the Sambrook et al. treatise of 1989.

For these reasons, the Examiner is requested to reconsider the rejection and to withdraw it.

G. Tissue construct comprising fibroblasts derived from tendon, lung, urethra, umbilical cord, corneal stroma, oral mucosa, and intestine, or a bilayer construct comprising corneal epithelial cells, or epithelial cells from oral mucosa, esophageal epithelial cells, and uroepithelial cells, or a method of transplantation or implantation in a patient.

The Examiner states that the specification does not enable a tissue construct comprising fibroblasts derived from tendon, lung, urethra, umbilical cord, corneal stroma, oral mucosa, and intestine, or a bilayer construct comprising corneal epithelial cells, or epithelial cells from oral mucosa, esophageal epithelial cells, and uroepithelial cells, or a method of transplantation or implantation in a patient.

Applicants respectfully traverse this rejection. It is within the range of routine experimentation to use fibroblast cells derived from different selected tissues. It would not require undue experimentation to make and use the tissue constructs of cell types other than fibroblasts from neonate foreskin and derma and keratinocytes, and to transplant said constructs into animals other than athymic mice. One of skill in the art would be able to use different sources of cells to practice this invention.

For these reasons, the Examiner is requested to reconsider and to withdraw this rejection.

II. Rejections Under Section 112, Second Paragraph.

Claims 1-30 are rejected for various reasons as being unclear under § 112, second paragraph. Applicants respectfully traverse these rejections. The individual rejections are discussed below.

A. Claims 1-30 are rejected under § 112, second paragraph, as being unclear as to the metes and bounds of "exogenous matrix components" and "synthetic members"

Exogenous matrix components are matrix components not produced by the cultured cells but introduced by other means. (page 4, lines 6-7).

Synthetic members are used for structure or support or both. (page 3, line 7). An example is a mesh member for the formation of the tissue constructs (page 10, lines 21-22).

For these reasons, the Examiner is requested to reconsider the rejections and to withdraw them.

B. Claims 7 and 21 are rejected under § 112, second paragraph, as being unclear as to the metes and bounds of "no non-human components."

The specification at page 2, lines 1-2 elucidates the phrase "no non-human components" by stating that the methods of the invention can be carried out "without the use of undefined or non-human-derived biological components, such as bovine serum or organ extracts." In addition, the specification at page 12, lines 1-8, says:

> When the invention is carried out utilizing screened human cells cultured using chemically defined components derived from no non-human animal sources, the resultant tissue construct is a defined human tissue construct. Synthetic functional equivalents may also be added to supplement chemically defined media within the purview of the definition of chemically defined for use in the most preferred fabrication method. Generally, one of skill in the art of cell culture will be able to determine suitable natural human, human recombinant, or synthetic equivalents to commonly known animal components to supplement the culture media of the invention without undue investigation or experimentation.

For these reasons, the Examiner is requested to reconsider and withdraw the rejection.

C. Claims 19-23 and 24-26 are rejected under § 112, second paragraph, as being unclear as to the steps involved in "stimulating" the fibroblast cells to synthesize, secrete, and organize extracellular matrix components.

Applicants respectfully submit that the steps involved in "stimulating" the fibroblast cells to synthesize, secrete, and organize extracellular matrix components are adequately defined in the specification and well-known to those of skill in the art. Thus, the specification at page 17, lines 7-9 (herein amended) states that "When the matrix producing cells are confluent, and the culture medium is supplemented with components that assist in matrix synthesis, secretion, or organization, the cells are said to be stimulated to form a tissue-construct comprised of cells and matrix synthesized by those cells." In addition, the specification indicates at page 6, line 13, that the culturing and stimulating steps may be done simultaneously or consecutively, thus indicating that the cells do not need to be confluent before stimulation. The specification further describes a preferred matrix production medium formulation as well as agents to up-regulate collagen transcription. (page 17, lines 10-24).

For these reasons, the Examiner is requested to reconsider and withdraw the rejection.

D. Claims 1-18, 28, and 30 are rejected under § 112, second paragraph, as being unclear as to the metes and bounds of cultured "under conditions to produce a layer of extracellular matrix."

It is well-established that the determination whether a claim is invalid as indefinite depends on whether those skilled in the art would understand the scope of the claim when the claim is read in light of the specification. One skilled in the art would understand the scope of

this claim language when read in light of the specification. For this reason, the Examiner is requested to reconsider and to withdraw this rejection.

III. Rejections Under Section 103.

A. Claims 1-3, 6-12, and 19-27 are rejected under Section 103 as obvious over Bell, U.S. 4,485,096, Parenteau et al., U.S. 5,712,163, Sand, U.S. 5,618,284, Holbrook et al. (1993) and Biegel et al. (1994).

Applicants respectfully traverse this rejection. Applicants are well acquainted with the cited references Bell and Parenteau as these patents are licensed and assigned, respectively, to the assignee of the instant application.

The patentable distinction between the Bell patent and the instant application is that Bell requires the use of a hydrated lattice or an exogenous matrix component.

Parenteau et al. does teach a chemically defined cell culture medium, but does not teach or suggest the instantly claimed invention.

Applicants disagree with the characterization of Parenteau et al. as teaching the absence of exogenous matrix components or synthetic membranes (Office Action, page 10, line 3-4) because Parenteau et al. points out the benefit of having a substrate present when culturing the cells. Parenteau et al. states that "[v]arious substrates can be used in the practice of the present invention . . . . In the claimed systems, growth may be enhanced under certain conditions using either fibronectin or collagen coated substrate . . . . The presence of a substrate, e.g., a matrix component appears to allow the cells to establish colonies when conditions are less than optimal or more stringent . . . ." (col. 18, line 63 to col. 19, line 19).

Sand states what is known in the art, that is, that human type-1 collagen molecule consists of chains of 300 nm triple helixes joined by 67 nm uncoiled bonds.

Holbrook et al. also state what is known in the art, that is that the dermal matrix of connective tissue is comprised of collagen (of which 80-90% is type I and 8-12% is type III) glycosaminoglycan, fibronectin, and tenascin.

Biegel et al. does describe coating transwell filters with hydrated collagen gels and then culturing endothelial cells. The hydrated collagen gel is similar in principle to the hydrated lattice of Bell, above, and is an exogenous matrix component.

The instantly claimed invention is directed to bioengineered tissue constructs of cultured cells and endogenously produced extracellular matrix components without the requirement of exogenous matrix components or synthetic members. The invention is not rendered obvious by the cited references. The Examiner is therefore requested to reconsider this rejection and to withdraw it.

B. Claims 1, 4, 5, 9, 13, and 14 are rejected under Section 103(a) as obvious over Jahoda et al. (1993) in view of Parenteau et al., U.S. 5,712,163.

Applicants respectfully traverse this rejection. Jahoda et al. teach that the transplantation of low passage dermal papilla cells in rat ear wounds resulted in the production of hair growth in comparison to a control of transplanted skin fibroblasts and later passage dermal papilla cells. In Jahoda et al., the cells are scraped from the base of a dish with a rubber policeman and removed in clumps which are then pushed into the wound gap. (page 585-586). Jahoda et al. does not teach the creation of a cultured tissue construct comprising cells grown under conditions to produce a layer of extracellular matrix which is synthesized and assembled by the cultured cells and wherein said extracellular matrix is produced by the cultured fibroblast cells in the absence of exogenous matrix components or synthetic support members during the culturing conditions.

Parenteau et al. teach the use of a chemically defined culture medium. Parenteau et al. do not show the presence in the extracellular matrix of fibrillar collagen showing a packing organization of fibrils and fibril bundles exhibiting a quarter-staggered 67 nm banding pattern, decorin, or glycosaminoglycans. Furthermore, Parenteau et al. do not show a cultured tissue construct comprising fibroblast cells grown under conditions to produce a layer of extracellular matrix which is synthesized and assembled by the cultured fibroblast cells or an extracellular matrix produced by the cultured fibroblast cells.

Applicants disagree with the characterization of Parenteau et al. as teaching the absence of exogenous matrix components or synthetic membranes (Office Action, page 12, line 7-8) because Parenteau et al. points out the benefit of having a substrate present when culturing the cells. Parenteau et al. states that "[v]arious substrates can be used in the practice of the present invention . . . . In the claimed systems, growth may be enhanced under certain conditions using either fibronectin or collagen coated substrate . . . . The presence of a substrate, e.g., a matrix component appears to allow the cells to establish colonies when conditions are less than optimal or more stringent . . . ." (col. 18, line 63 to col. 19, line 19).

Thus, it would not have been obvious to one of ordinary skill in the art at the time of the invention to create a cultured tissue construct comprising fibroblast cells grown under conditions to produce a layer of extracellular matrix which is synthesized and assembled by the cultured cells and wherein said extracellular matrix is produced in the absence of exogenous matrix components or such a construct wherein said cultured cells are dermal papilla cells with or without a top layer of epithelial cells.

IV. Conclusion.

Applicants respectfully submit that all the bases for rejection of the pending claims are now moot. The Examiner is requested to reconsider the rejections and to withdraw them and to pass this case to issuance.

Respectfully submitted,

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Marked Up Version of Replacement Paragraphs in Specification Under 37 C.F.R. §1.121 (b)(1)(iii)

Paragraph on page 5, lines 3-25:

While the aforementioned list is a list of biochemical and structural features **in** a cultured cell-matrix construct formed from dermal fibroblasts, it should be recognized that cultured cell-matrix constructs formed from other types of fibroblasts will produce many of these features and others phenotypic for **a** tissue type from which they originated. In some cases, fibroblasts can be induced to express non-phenotypic components by either chemical exposure or contact, physical stresses, or by transgenic means. Another preferred embodiment of the invention is a cell-matrix layer having **a** second layer of cells disposed thereon. The second layer of cells is cultured on the cell-matrix layer to form a bioengineered bilayered tissue construct. In a more preferred embodiment, the cells of the second layer are of epithelial origin. In the most preferred embodiment, the second layer comprises cultured human keratinocytes that together with a first cell-matrix layer, a cell-matrix construct formed from dermal fibroblasts and endogenous matrix to form a dermal layer, comprise a living skin construct. When fully formed, the epidermal layer is a multilayered, stratified, and well-differentiated layer of keratinocytes that exhibit a basal layer, a suprabasal layer, a granular layer and a stratum corneum. The skin construct has a well-developed basement membrane present at the dermal-epidermal junction as exhibited by transmission electron microscopy (TEM). The basement membrane appears thickest around hemidesmosomes, marked by anchoring fibrils that are comprised of type VII collagen, as visualized by TEM. The anchoring fibrils can seen exiting from the basement membrane and entrapping the collagen fibrils in the dermal layer. These anchoring fibrils, as well as other basement membrane components, are secreted by keratinocytes. It is also known that while keratinocytes are capable of secreting basement membrane components on their own, a recognizable basement membrane will not form in the absence of fibroblasts. Immunohistochemical staining of the skin construct of the present invention has also shown that laminin, a basement membrane protein is present.

Paragraph on page 7, lines 20-27:

Although human cells are preferred for use in the invention, the cells to be used in the method of the **invention** are not limited to cells from human sources. Cells from other mammalian species including, but not limited to, equine, canine, porcine, bovine, and ovine sources; or rodent species such as mouse or rat may be used. In addition, cells that are spontaneously, chemically or virally transfected or recombinant cells or genetically engineered cells may also be used in this invention. For those embodiments that incorporate more than one cell type, chimeric mixtures of normal cells from two or more sources; mixtures of normal and genetically modified or transfected cells; or mixtures of cells of two or more species or tissue sources may be used.

Paragraph on page 10, lines 21-29:

The cultured tissue constructs of the invention do not rely on synthetic or bioresorbable members ~~for~~, such as a mesh member for the formation of the tissue constructs. The mesh member is organized as a woven, a knit, or a felt material. In systems where a mesh member is employed, the cells are cultured on the mesh member and growing on either side and within the interstices of the mesh to envelop and incorporate the mesh within the cultured tissue construct. The final construct formed by methods that incorporate such a mesh rely on it for physical support and for bulk. Examples of cultures tissue constructs that rely on synthetic mesh members are found in U.S. Patent Numbers 5,580,781, 5,443,950, 5,266,480, 5,032,508, 4,963,489 to Naughton, et al.

Paragraph on pages 16-17, lines 20-30 and 1-6:

While not required, the matrix-production medium is optionally supplemented with a neutral polymer. The cell-matrix constructs of the invention may be produced without a neutral polymer, but again not wishing to be bound by theory, its presence in the matrix production medium may **assist in** collagen processing and deposition more consistently between samples. One preferred neutral polymer is polyethylene glycol (PEG), which has been shown to promote in vitro processing of the soluble precursor procollagen produced by the cultured cells to matrix deposited collagen. Tissue culture grade PEG within the range between about 1000 to about 4000 MW (molecular weight), more preferably between about 3400 to about 3700 MW is preferred in the media of the invention. Preferred PEG concentrations ~~are~~ for use in the method may be at concentrations at about 5% w/v or less, preferably about 0.01% w/v to about 0.5% w/v, more preferably between about 0.025% w/v to about 0.2% w/v, most preferably about 0.05% w/v. Other culture grade neutral polymers such dextran, preferably dextran T-40, or polyvinylpyrrolidone (PVP), preferably in the range of 30,000-40,000 MW, may also be used at concentrations at about 5% w/v or less, preferably between about 0.01% w/v to about 0.5% w/v, more preferably between about 0.025% w/v to about 0.2% w/v, most preferably about 0.05% w/v. Other cell culture grade and cell-compatible agents that enhance collagen processing and deposition may be ascertained by the skilled routineer in the art of mammalian cell culture.

Paragraph on page 17, lines 7-9:

When the ~~cell~~ **matrix** producing cells are confluent, and the culture medium is supplemented with components that assist in matrix synthesis, secretion, or organization, the cells are said to be stimulated to form a tissue-construct comprised of cells and matrix synthesized by those cells.

Paragraph on pages 19-20, lines 26-30 and 1-11:

In the method of forming a differentiated epidermal layer, subcultured keratinocytes are taken from the cell stock and their cell numbers are expanded. When ~~an~~ **a** necessary number of cells have been obtained, they are released from the culture substrate, suspended, counted, diluted and then seeded to the top surface of the cell-matrix construct at a density between about 4.5×10^3 cells/cm^2 to about 5.0×10^5 cells/cm^2, more preferably between about 1.0×10^4 cells/cm^2 to about 1.0×10^5 cells/cm^2, and most preferably at about 4.5×10^4 cells/cm^2. The constructs are then incubated for between about 60 to about 90 minutes at $37 \pm 1^\circ$C, 10% CO_2 to allow the keratinocytes to attach. After the incubation, the constructs are submerged in epidermalization medium. After a sufficient length of time in culture, the keratinocytes proliferate and spread to form a confluent monolayer across the cell-matrix construct. Once confluent, the cell media formulation is changed to differentiation medium to induce cell differentiation. When a multilayer epithelium has formed, cornification media is then used and the culture is brought to the air-liquid interface. For the differentiation and cornification of keratinocytes, the cells are exposed to a dry or low humidity air-liquid interface. A dry or low-humidity interface can be characterized as trying to duplicate the low moisture levels of skin. With time, keratinocytes will express most or all keratins and other features found in native skin when exposed to these conditions.

Paragraph on pages 20-21, lines 12-30 and 1-11:

As mentioned above, the system for the production of a cell-matrix construct may be used in the formation of a corneal construct. The corneal epithelial cells can be derived from a variety of mammalian sources. The preferred epithelial cell is a rabbit or human corneal epithelial cell (corneal keratinocyte) but any mammalian corneal keratinocyte may be used. Other epithelial keratinocytes such as those derived from the sclera (outer white opaque portion) of the eye or epidermis may be substituted, but corneal keratinocytes are preferable. In the method for forming a corneal construct, the medium is removed from the culture insert (containing the cell-matrix construct) and its surround. Normal rabbit corneal epithelial cells -are expanded via subculture, trypsinized to remove them from the cultures substrate, suspended in culture medium, and seeded on top of the membrane at a density between about 7.2×10^4 to about 1.4×10^5 cells/cm^2. The constructs are then incubated without medium for about four hours at $37 \pm 1^{\circ}$C, 10% CO_2 to allow the epithelial cells to attach. After incubation, the constructs are submerged in Corneal Maintenance Medium (CMM) (Johnson et al., 1992.) The epithelial cells are cultured until the cell-matrix construct is covered with the epithelial cells. Completeness of epithelial coverage can be ascertained by a variety of methods, for illustration by staining the culture with a solution of Nile Blue sulfate (1:10,000 in phosphate buffered saline). Once the cell-matrix construct is covered, after approximately seven days, the constructs are aseptically transferred to new culturing trays with sufficient ~~cornea~~ **corneal** maintenance medium (CMM) to achieve a fluid level just to the surface of the construct to maintain a moist interface without submersion of the epithelial layer. The constructs are incubated at $37 \pm 1^{\circ}$C, 10% CO_2, and greater than 60% humidity, with the CMM, making media changes, as necessary, typically, three times per week.

Paragraph on page 21, lines 12-25:

In an alternate preferred embodiment, a seeding of a second matrix-producing cell may be performed on a first formed cell-matrix construct to obtain a thicker cell-matrix construct or a bilayer cell-matrix construct. The second seeding can be performed with the same cell type or strain or with a different cell type or strain, depending on the desired result. The second seeding is performed under the same conditions employing the procedures and matrix production medium used in the production of the first layer. One result in performing the second seeding with a different cell type is to have a matrix formed with different matrix component profiles or matrix packing density to affect wound healing when the construct is grafted to a patient. The first cell seeding produces a matrix analogous to the reticular layer of dermis, a more densely packed layer of Type I collagen and constituent extracellular matrix components. The second cell seeding would ~~produces~~ **<u>produce</u>** a matrix similar to the papillary layer of dermis characterized by looser collagen fibrils and extracellular matrix. Another result is **<u>that</u>** the second cell type may produce a therapeutic substance that would also affect wound healing, such as improved graft take or graft integration or the minimization or prevention of scar formation.

Paragraph on pages 21-22, lines 26-30 and 1-15:

In another preferred embodiment, mixed cell populations of two or more cell types may be cultured together during the formation of a cell-matrix construct provided that at least one of the cell types used is capable of synthesizing extracellular matrix. The second cell type may be one needed to perform other tissue functions or to develop particular structural features of the tissue construct. For instance, in the production of a skin construct, dermal papilla cells or epithelial cells from adnexas may be cultured with the matrix-producing cells to allow the formation of epithelial appendages or their components. Epidermal appendages such as sweat or sebaceous gland structures or components or hair follicle structures or components may form when cultured together with the matrix-producing cells. Epithelial cells may be derived from the appendageal structures of gland and hair located in deep dermis, such as by microdissection, and include eccrine cells, myoepithelial cells, glandular secretory cells, hair follicle stem cells. Other cell types normally found in skin that constitute skin may also be added such as melanocytes, Langerhans cells, and Merkel cells. Similarly, vascular endothelial cells may be co-cultured to produce rudimentary components for new vasculature formation. Adipocytes may also be cultured with the matrix-producing cells to form a construct used for reconstructive surgery. As alternate mode of delivery of this second cell type, the cells may **be** locally seeded as a spot or as an arrangement of any number of spots of cells on or within a forming or completely formed cell-tissue matrix for localized development of these structures. To seed the cells within the cell-matrix construct, the cells may be injected between the top and bottom surfaces, within the cell-matrix, for the cells to grow, form specialized structures and perform their specialized function**.**

Paragraph on page 35, lines 6-22:

Cell-matrix constructs were prepared according to the methods in Example 1 using human dermal fibroblasts derived from neonate foreskin and were grafted onto full excision wounds created on nude athymic mice. Mice were grafted according to the methods described by Parenteau, et al. (1996), the disclosure of which is incorporated herein. Grafts were examined at 14, 28 and 56 days for signs of adherence to the wound bed, evidence of wound contraction, areas of graft loss, and presence of vascularization (color). The graft areas were photographed while intact on the mice. A number of mice were sacrificed at each timepoint, and the graft areas and their surrounds were excised along with a surrounding rim of murine skin to at least the panniculus carnosus. Junctions between the graft and the murine skin were preserved in each sample. The explanted tissue samples were then fixed in phosphate buffered 10% formalin and fixation in methanol. Formalin fixed samples were processed for H&E staining according to procedure described in Example 1. Grafts were able to integrate with the mouse skin, with minimal contraction noted. Within 14 days of grafting, the mouse epidermis had migrated completely over the graft. Using the H&E stained samples, vessels were obvious within the graft at 14 days, and throughout the experiment. By gross observation and by H&E stained samples, it was determined that the graft persisted and remained healthy looking (contained living cells, no gross matrix abnormalities, etc.) throughout the length of the experiment.

Paragraph on page 45, lines 14-28:

Samples were formalin fixed and processed for hemotoxylin and eosin staining for light microscope analysis. Visual histological evaluation demonstrated that the Condition 2 lacking PEG demonstrated a comparably similar matrix as Condition 1 containing PEG. Biochemical analysis measuring the collagen content of the construct showed nearly the same amount of collagen in both: 168.7 ± 7.98 $\mu g/cm^2$ for Condition 1 with PEG as compared to 170.88 ± 9.07 $\mu g/cm^2$ for Condition 2 without PEG. Condition 3 containing high levels of insulin and hydrocortisone showed a higher expression of matrix, including collagen, at a timepoint earlier than the other two conditions. Besides endogenously produced fibrillar collagen, decorin and glycosaminoglycan were also present in the cell-matrix constructs in all Conditions. The cultured dermal construct formed by the method of Condition 2 of this Example is shown in Figure 2. Shown in Figure 2 is a photomicrograph of a fixed, paraffin embedded, hematoxylin and eosin stained section of a cell-matrix construct formed from cultured human dermal fibroblasts in chemically defined medium at 21 days. The porous membrane appears as a thin translucent band below the construct and it can be seen that the cells grow on the surface of the membrane and do not envelope ~~in~~ **or** integrate the membrane with matrix.

On page 46, please delete the paragraph at lines 12-15 and replace it with the following paragraph:

Using a 21 day dermal construct formed by human dermal fibroblasts under chemically defined conditions according to the method of Condition 2 (without PEG) described in Example **15, above,** normal human neonatal foreskin epidermal keratinocytes were seeded on the top surface of the cell-matrix construct to form the epidermal layer of the skin construct.